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Pax6 autoregulation mediated by direct interaction of Pax6 protein with the head surface ectoderm-specific enhancer of the mouse *Pax6* gene

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Abstract

The *Pax6* gene plays crucial roles in eye development and encodes a transcription factor containing both a paired domain and a homeodomain. During embryogenesis, *Pax6* is expressed in restricted tissues under the direction of distinct *cis*-regulatory regions. The head surface ectoderm-specific enhancer of mouse *Pax6* directs reporter expression in the derivatives of the ectoderm in the eye, such as lens and cornea, but the molecular mechanism of its control remains largely unknown. We identified a Pax6 protein-responsive element termed LE9 (52 bp in length) within the head surface ectoderm-specific enhancer. LE9, a sequence well conserved across vertebrates, acted as a highly effective enhancer in reporter analyses. Pax6 protein formed in vitro a complex with the distal half of LE9 in a manner dependent on the paired domain. The proximal half of the LE9 sequence contains three plausible sites of HMG domain recognition, and HMG domain-containing transcription factors Sox2 and Sox3 activated LE9 synergistically with Pax6. A scanning mutagenesis experiment indicated that the central site is most important among the three presumptive HMG domain recognition sites. Furthermore, Pax6 and Sox2 proteins formed a complex when they were expressed together. Based on these findings, we propose a model in which Pax6 protein directly and positively regulates its own gene expression, and Sox2 and Sox3 proteins interact with Pax6 protein, resulting in modification of the transcriptional activation by Pax6 protein.

Keywords: Lens development; Placode formation; Transcription factor; Gene expression; DNA-binding protein; Sequence recognition; Paired domain; HMG domain; Tissue-specific enhancer

Introduction

Pax6 encodes a member of the Pax family transcription factors and has been identified as a key regulator gene of eye development in both vertebrates and invertebrates (Walther et al., 1991; Halder et al., 1995). During vertebrate development, the *Pax6* gene is expressed in the eye, the central nervous system, some of the sensory placodes, and the pancreas (Grindley et al., 1995; Callaerts et al., 1997; Mansouri et al., 1999). Transgenic studies in mice using the *lacZ* reporter gene identified, in the upstream regulatory region of the mouse *Pax6* gene, a modular structure of the enhancers responsible for various expression domains (Williams et al., 1998; Kammandel et al., 1999; Xu et al., 1999); four distinct enhancer regions have been identified thus far for the pancreas, the head surface ectoderm, the telencephalon, and the retina, respectively. The head surface ectoderm-specific enhancer directs the reporter gene expression in a spatiotemporal pattern similar to that of the endogenous *Pax6* expression in the derivatives of the head surface ectoderm in the eye, i.e., the developing lens, cornea, conjunctiva, and lacrimal gland (Kammandel et al., 1999; Williams et al., 1998). In particular, the central 102-bp region of the enhancer has been found essential for the lens-specific expression of the reporter gene (Kammandel et al., 1999). The sequence of the enhancer is highly conserved among vertebrates, and disruption of the enhancer causes abnormalities in lens development (Dimanlig et al., 2001).

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Pax6 plays essential roles in lens development, and its expression level has critical effects on this process. Studies of *Pax6* overexpression (Schedl et al., 1996; Chow et al., 1999) and analyses of haploinsufficiency of the *Small eye (Sey)* mutant mouse, which carries a mutation in the *Pax6* gene (Hogan et al., 1988; Hill et al., 1991; van Raamsdonk and Tilghman, 2000), have provided evidence that altered levels of *Pax6* expression result in defects in lens development. The endogenous *Pax6* transcript is initially detected in a broad area of the head surface ectoderm at earlier stages than the emergence of the lens placode. Later, the expression is more concentrated in the lens placode and the lens vesicle, and is also detected in the surface ectoderm over the lens vesicle, which gives rise to the cornea and the conjunctiva (Li et al., 1994; Grindley et al., 1995). The mechanism of control of the level of *Pax6* expression in the developing lens is largely unknown, but is considered to be complex and multistep. It was demonstrated by studies of the level of expression of the *Pax6* transcript in *Sey/Sey* mouse embryos that *Pax6* autoregulates its own expression in the presumptive lens ectoderm (Grindley et al., 1995). It was uncertain, however, whether this autoregulation was mediated in a cell-autonomous, or in a non-cell-autonomous fashion, since normal lens development is critically dependent on the tissue interaction between the prospective lens ectoderm and the optic vesicle, where the *Pax6* gene is also expressed. Recently, a study of conditional gene disruption of mouse *Pax6* in the prospective lens ectoderm revealed that Pax6 protein itself is required in the ectoderm for sustained *Pax6* gene transcription (Ashery-Padan et al., 2000). These in vivo studies provided evidence that Pax6 protein is cell-autonomously required for its own gene expression in the developing lens, but the molecular basis for the *Pax6* autoregulation is not yet fully understood.

In this study, we attempted to elucidate the mechanism of control of *Pax6* gene expression by examining the head surface ectoderm-specific enhancer of the mouse *Pax6* gene. We found that the enhancer is activated by Pax6 and Sox proteins in reporter assays, and that Pax6 alone exhibits substantial activation of the enhancer. We identified a Pax6-responsive element within this enhancer region. Our results suggest that Pax6 protein directly recognizes this element via the paired domain and interacts with Sox2 and Sox3 proteins to synergistically activate the enhancer element. Based on the present results, we propose a model in which the *Pax6* gene directly regulates its own expression in the lens region during development.

Materials and methods

Cell culture

SRA01/04 human lens epithelial cells were established and maintained as previously described (Ibaraki et al., 1998). NMuMG mouse mammary gland epithelial cells and RL-34 rat liver epithelial cells were obtained from American Type Culture Collection and Science Research Resources Bank, Japan, respectively, and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum.

Construction of reporter and effector plasmids

Construction of the control plasmid pRL-1PGK, which carries the constitutive promoter of the mouse *Pgk-1* gene (Boer et al., 1990) and the *Renilla* luciferase cDNA, and the reporter plasmid pE4luc, which contains the adenovirus E4 promoter and the firefly luciferase cDNA, were described previously (Kitamura et al., 2000). The *Pax6* P0 promoter was obtained by PCR using oligonucleotides 197(GCACTCGAGGCAACCAATGAGGGCATT) and 156(ACCAAGCTTGACAACCGGGTTCTACGCGAGGA) as PCR primers, and mouse genomic DNA as template. The 129-bp fragment was inserted between the *Xho*I–*Hin*dIII sites of pGL3-basic plasmid (Promega), and the resulting plasmid was termed pP0luc. The mouse *Pax6* head surface ectoderm-specific enhancer region (Williams et al., 1998; Kammandel et al., 1999) was also cloned by PCR. For making reporter plasmids, various fragments of the enhancer region were cloned between the *Sac*I and *Xho*I sites of pE4luc and pP0luc. The position of each enhancer fragment was as follows: LE4, from the 1st to 526th base sequence of GenBank entry MMPAXP1 (Accession No. AF098639); LE4d, 1–173 and 305–526; LE3, 1–341; LE1, 166–341; LE2, 166–309; LE6, 166–244; LE7, 196–309; LE8, 236–309; LE12, 175–244; LE9, 166–217; LE10, 188–217; LE11, 166–195; LE0, 166–309.

As controls, we also constructed pPRD and pPRDx4 reporter plasmids, which carry an "optimal" binding sequence of the Pax6 paired domain (CATTTTCACGCATGAGTGCACA; Epstein et al., 1994a) at the *Xho*I site of pE4luc. The latter plasmid contains four repeats of this binding sequence. All constructs were verified by DNA sequencing.

A cDNA clone encoding human Pax6 isoform b (also called Pax6-5a) was obtained from the IMAGE consortium EST project (Lennon et al., 1996). The extra exon 5a of *Pax6b* was removed by means of site-directed mutagenesis, and the resultant fragments were inserted in a constitutive expression vector, pactEF (Okazaki and Sagata, 1995). The entire coding regions of mouse cDNAs for Sox2 and Sox3 were also similarly cloned in pactEF.

DNA transfection

For transfection of cells seeded in a 12-well plate, 80 ng of pRL-1PGK and 270 ng of each firefly luciferase reporter were combined with various amounts of effector plasmids. Control plasmid pactEF was included to make the total amount of DNA 500 ng. Fugene 6 transfection reagent (Roche, 1.25 μl) was added to each DNA mixture in 60 μl

of DMEM. After 30 min of incubation at room temperature, the DNA–Fugene complex was added to each well. After 24 h, firefly and *Renilla* luciferase activities in the cell lysate were measured by using a luminometer equipped with two injectors for the Dual-luciferase assay reagents (Promega). Relative luciferase activity was calculated by dividing the level of firefly luciferase activity by the level of *Renilla* luciferase activity. DNA was independently prepared at least twice, and the assay for each reporter construct was repeated four times.

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides were synthesized based on the sequences of the enhancer fragments. An extra "CG" dinucleotide sequence was attached at the 5′ end of each oligonucleotide sequence for 3′ fill-in labeling with [α-^{32}P]dCTP by the Klenow fragment of *Escherichia coli* DNA polymerase I. For in vitro synthesis of Pax6 and Pax6b, cDNA fragments cloned in pT7G plasmid (Oe et al., 2001) were linearized and incubated in a phage T7 RNA polymerase-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions.

Nuclear extracts were prepared as follows. NMuMG cells were transfected with the Pax6 expression plasmid (12 μg) and Fugene 6 (24 μl) in 9-cm-diameter dishes. After 24 h, the cells were washed twice with ice-cold PBS and then with hypotonic buffer (10 mM Tris–HCl, pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl). The cells were lysed in 1 ml of cell-lysis buffer (10 mM Tris–HCl, pH 7.4 at 25°C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 7 mM 2-ME, 0.1% NP-40) on ice, and the total cell lysate was collected by scraping into a 1.5-ml centrifuge tube. Cell nuclei were obtained by centrifugation at 800*g* for 2 min at 4°C, and then incubated in extraction buffer (10 mM Tris–HCl, pH 7.4 at 25°C, 20 % glycerol, 340 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.1% 2-ME) for 40 min on ice. Nuclear extracts were prepared by centrifuging the mixture at 12,000*g* for 15 min at 4°C.

For EMSA, the nuclear extracts were diluted about 30-fold (to 20 μg/ml protein concentration) with dilution buffer (20 mM Hepes–KOH, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.1% NP-40, 7 mM 2-ME, 0.5 mg/ml BSA, 0.5 mM PMSF). Labeled probe (5000 cpm, about 2–5 $\times 10^{-15}$ mol) was mixed with 1 μl of the diluted extract, 5 μl of 2$\times$ binding buffer (40 mM Hepes–KOH buffer, pH 7.9, 30% glycerol, 200 mM KCl, 14 mM 2-ME), and 0.5 μg (unless otherwise noted) of poly(dI–dC) (Amersham-Pharmacia) in a total volume of 10 μl, and incubated at room temperature for 30 min. For super-shift analysis, 1 μg of monoclonal antibody for Pax6 protein or nonimmune IgG was included. Electrophoresis was performed by using 6% acrylamide gels and 0.5$\times$ TBE buffer.

For the EMSA for Sox2 binding, recombinant proteins containing the HMG domain of Sox2 (amino acid residues 1–130) or the paired domain of Pax6 (1–136) was purified by using the GST system (Amersham-Pharmacia). Labeled probe was mixed with 1 μl of diluted recombinant proteins in the dilution buffer supplemented with 0.2 mg/ml BSA (see above) and 5 μl of 2$\times$ binding buffer in a total volume of 10 μl.

Immunoprecipitation and immunoblotting

Pax6- and Sox2-coding sequences were N-terminally tagged with Flag and Myc epitope tags, respectively.

Transfection of 293T cells and preparation of the cell lysate under nondenaturing conditions were previously described (Kitamura et al., 2000; Okazaki and Sagata, 1995). Immunoprecipitation was carried out by using anti-Flag M2 antibody (KODAK) or anti-Myc 9E10 antibody (1 μg each) and 30 μl of immobilized protein A beads (Pierce) for each cell lysate (300 μg of protein), and half of the immunoprecipitated proteins were used for SDS–PAGE. After SDS–PAGE, the proteins were immunoblotted by using the anti-Flag or anti-Myc antibody as previously described (Kitamura et al., 2000). HRP-linked anti-mouse κ-light chain was used as the secondary antibody.

Results

Identification and mapping of a Pax6-responsive element in the head surface ectoderm-specific enhancer of the Pax6 gene

To assess activation by Pax6 protein, we first transfected SRA01/04 human lens epithelial cells with luciferase reporter plasmids carrying different portions of the head surface ectoderm-specific enhancer region of the mouse *Pax6* gene (Fig. 1A and B) together with various amounts of the Pax6-expression vector (Fig. 1C). Among the tested DNA fragments, LE0 corresponds to the minimal lens-specific enhancer region determined in a transgenic study (Kammandel et al., 1999). We also prepared the PRD reporter construct, which contains an "optimal" binding sequence of the Pax6 paired domain (Epstein et al., 1994a), as a control.

As shown in Fig. 2A, several enhancer fragments, such as LE9 and LE6, responded to Pax6 protein in a dose-dependent manner. When epithelial cells derived from tissues other than the lens were used, Pax6 coexpression also activated reporter constructs carrying the LE9 sequence (Fig. 2B for RL-34 rat liver epithelial cells; similar data obtained with NMuMG mouse mammary gland epithelial cells are not shown), indicating that this activation by Pax6 protein occurred in various cell types. Although SR01/04 cells retain some characteristics of lens epithelial cells (Ibaraki et al., 1998), the expression level of Pax6 in SR01/04 cells was below the limit of detection of Western blotting (data not shown) using an anti-Pax6 monoclonal antibody (Kawakami et al., 1997).

In these reporter assays, Pax6 protein only weakly acti-

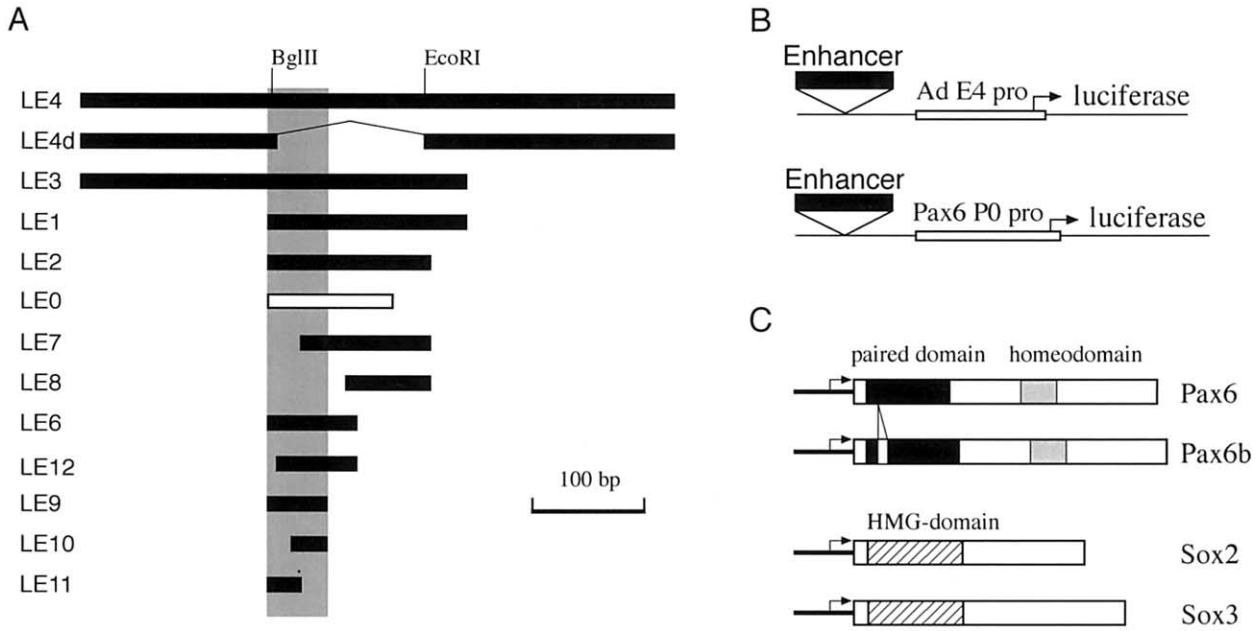

Fig. 1. Mapping a Pax6 protein-responsive site upstream of the mouse *Pax6* gene. (A) A schematic diagram of a series of partially overlapping DNA fragments that encompass the head surface ectoderm-specific enhancer region of the mouse *Pax6* gene. The head surface ectoderm-specific enhancer region, LE4, has a sequence highly conserved between human and mouse (Williams et al., 1998). LE0, indicated by an open box, corresponds to the minimal region for the lens-specific enhancer activity determined in a transgenic mouse study (Kammandel et al., 1999). The region found to have full activity in the present study (LE9) is shaded in gray for easy comparison of the positions of the fragments. (B) The structures of the transcription reporter plasmids. Each enhancer fragment was inserted into the reporter vectors with the coding sequence for firefly luciferase and either the adenovirus E4 promoter or the mouse *Pax6* P0 promoter. (C) The expression vectors for Pax6 and Sox proteins. Coding sequences for Pax6, Pax6b, Sox2, and Sox3 were driven by a constitutive promoter in pactEF expression vector. The regions encoding the paired domain and the homeodomain of Pax6 are shown as black and gray boxes, respectively. The cDNA for Pax6b contains an insertion (shown by a white box) derived from alternatively spliced exon 5a in the paired domain. The HMG domains of Sox2 and Sox3 are indicated by hatched boxes.

vated reporter plasmids containing a single copy of PRD or LE11 (a subregion of LE9; Fig. 1). The reporter plasmid with the LE11 sequence in the reverse orientation (LE11R), however, appeared to respond to Pax6 (Fig. 2C). Among the reporter constructs with four repeats of enhancer fragments, PRDx4 and LE11x4, but not LE10x4, was activated by Pax6 in RL-34 cells (Fig. 2D). Similar results were obtained with other types of cells (data not shown). LE9 consistently showed Pax6-responsiveness in either orientation. Taken together, these results indicate that the LE9 fragment of the head surface ectoderm-specific enhancer region acts as a transcriptional enhancer when sufficient levels of Pax6 protein are present in various cell types, including lens cells. The distal half of LE9 (LE11) exhibited Pax6-response in some reporter constructs, suggesting that this part of LE9 is important for Pax6-responsiveness. Interestingly, LE6 and LE9 responded to Pax6 better than longer fragments, such as LE3 and LE4, that included the whole LE9 region (Fig. 1). This result suggests that there may be negative regulatory elements within the enhancer sequence but outside of LE9.

The mouse *Pax6* gene has three distinct promoter regions, P0, P1, and Pα (Kammandel et al., 1999), and an in situ hybridization study using exon-specific probes indicated that the P0-initiated transcripts are prominently expressed in the lens region during mouse development (Xu et al., 1999). Furthermore, the sequence of the P0 promoter region is highly conserved between mouse and quail. It has been reported that quail Pax6 protein can bind to its own P0 promoter, although the isolated P0 promoter alone is not sufficient to induce a cell type-specific response in a reporter assay (Plaza et al., 1993). We therefore examined a mouse *Pax6* P0 promoter fragment which contains the region corresponding to the quail Pax6-binding candidate site. Reporter assays using the adenovirus E4 promoter and the mouse *Pax6* P0 promoter showed similar responses to Pax6 protein (Fig. 2E). The effect of LE9 on transcriptional activation was much larger than the difference of activity between these two promoters. For simplicity, we describe only the results of reporter assays using constructs with the E4 promoter in the following experiments.

We next examined the Pax6b (also called Pax6-5a) isoform which contains 14 extra amino acid residues derived from alternative splicing in its paired domain. In sharp contrast to Pax6, Pax6b coexpression resulted in no notable activation of the LE9 reporter (Fig. 2F). Since the insert in the Pax6b paired domain causes alteration of the DNA binding specificity (Epstein et al., 1994b; Kozmik et al., 1997), our results strongly suggest that the paired domain of Pax6 is involved in the activation of LE9 and LE11 by Pax6 protein.

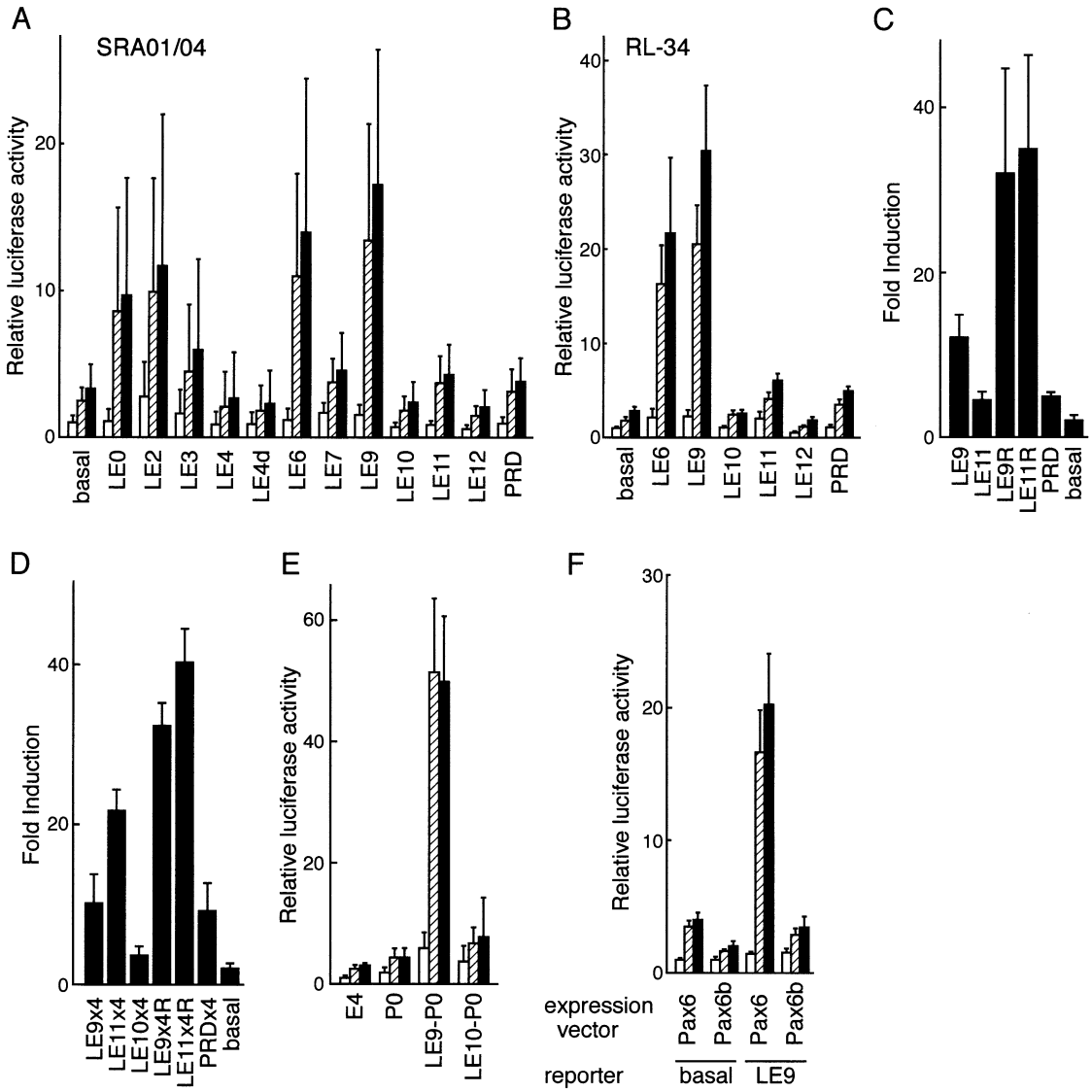

Fig. 2. LE9 acts as a minimum Pax6-responsive element. (A) Transcriptional activation of enhancer fragments in cultured lens cells. SRA01/04 lens epithelial cells were transfected with reporter plasmid containing the indicated fragment together with increasing amounts of Pax6 expression vector, pactEF-Pax6 (0, 75, or 150 ng; white, hatched, and black columns, respectively). Relative luciferase activities are shown as the average and standard deviation from four separate experiments. The activity of the basal reporter (pE4luc) in the absence of Pax6 expression vector was taken as 1. We also included an "optimal" binding sequence for the Pax6 paired domain as a control (PRD). These reporter plasmids utilize the adenovirus E4 promoter. The scores of Student's *t* test of LE9 and LE11 induction by Pax6 against the basal construct were $P < 0.001$ and $P = 0.23$, respectively, the latter of which was not significant. (B) Pax6-responsive enhancer activity in RL-34 cells. RL-34 liver epithelial cells were transfected and the luciferase activities were assayed as in (A). (C) LE11 was inserted in the reverse orientation (LE11R) and assayed in RL-34 cells. Activities are shown as fold-induction, which was calculated by dividing the level of the relative luciferase activity in the presence of Pax6 expression vector pactEF-Pax6 (150 ng) by that in the absence of the Pax6 expression vector. (D) Pax6-response of repeated enhancer sequences. LE9, LE10, or LE11 sequences were tandemly repeated four times, and inserted into the basal pE41uc reporter plasmid. "R" denotes the reverse orientation. Activities obtained with RL-34 cells are shown in fold-induction. (E) LE9 confers responsiveness to Pax6 protein on the *Pax6* P0 promoter and on the viral E4 promoter to a similar extent. The relative luciferase activity of the basal E4 reporter in the absence of cotransfection of Pax6 expression vector was taken as 1. The SRA01/04 cells were used. (F) Very weak effect of Pax6b isoform on LE9. The transactivational effect of Pax6b on LE9 was compared with that of Pax6. The relative luciferase activity of the basal reporter (pE4luc) without cotransfection of Pax6 expression vector was taken as 1.

Pax6 forms a complex with the distal half of LE9

To examine the physical interaction between the Pax6-responsive element and Pax6 protein, we performed EMSA using ^{32}P-labeled LE9 and LE11 probes (Fig. 3A). As shown in Fig. 3B, nuclear extract prepared from NMuMG cells transfected with the Pax6 expression construct markedly shifted the LE11 probe. Similar results were obtained with the LE9 probe, which includes the LE11 sequence (data not shown). When an anti-Pax6 monoclonal antibody (Kawakami et al., 1997) was added to the incubation mixture, a super-shift band appeared (Fig. 3B, lane 10). These

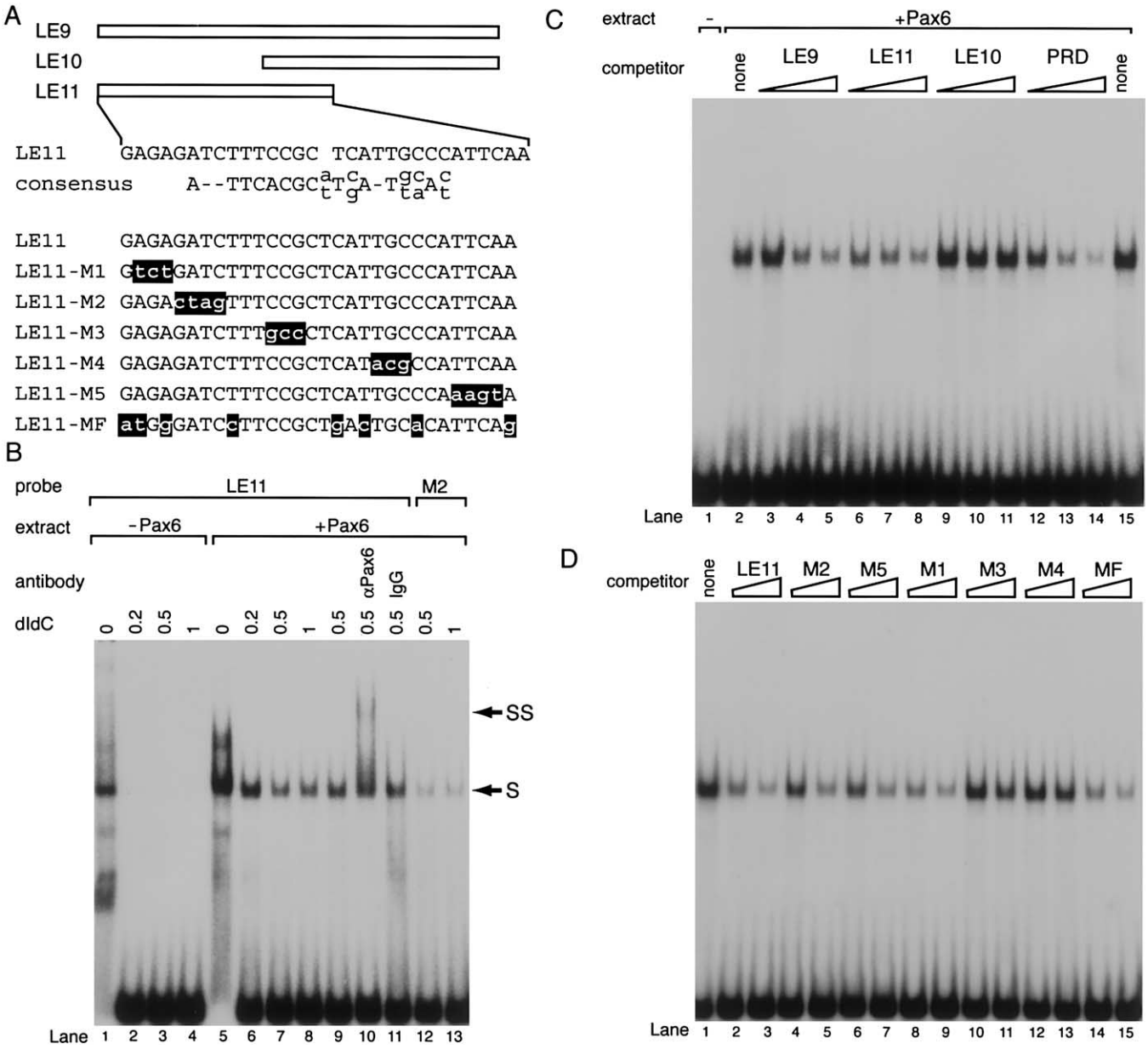

Fig. 3. Pax6 protein binds to the distal half of LE9. (A) Sequence similarity of Pax6 paired domain-binding consensus and the LE11 sequence. A schematic diagram of overlapping LE9, LE10, and LE11 fragments is shown by using open boxes. The sequence of LE11 and the consensus for the Pax6 paired domain binding sequences (Epstein et al., 1994a) are presented in an alignment. Base substitutions (from M1 to MF) introduced into the LE11 sequence are shown by white letters on a black background. LE11-MF contains the corresponding sequence from the fugu (pufferfish) *Pax6* gene. (B) Complex formation with LE11 and Pax6 protein. Pax6 protein-containing nuclear extracts were tested with the indicated probes in an electrophoretic mobility shift assay. The arrow marked "S" indicates a shifted band of the LE11 probe. Similar results were obtained with an LE9 probe (data not shown). The arrow marked "SS" indicates a supershift band that appeared as a result of coincubation with anti-Pax6 monoclonal antibody. (C) Competition assay for the Pax6–LE11 complex. Increasing amounts of the indicated DNA fragments (0.04, 0.1, and 0.2×10^{-12} mol) were included as unlabeled competitors with the labeled LE11 probe. "PRD" indicates the oligonucleotides with an optimal paired-domain binding sequence for Pax6 protein. (D) Competition assay using the mutated LE11 sequences (see A). The indicated individual LE11 mutants (0.1 and 0.2×10^{-12} mol) were examined as competitors to the labeled LE11 probe for binding to Pax6 protein.

results indicate that Pax6 protein forms a complex with DNA containing the LE11 sequence. In contrast, when nuclear extracts containing Pax6b protein were used, we observed no detectable specific protein–DNA complex (data not shown). When Pax6 or Pax6b protein synthesized using a cell-free in vitro translation system was used in the complex formation assay, essentially identical results to those with nuclear extracts were obtained (data not shown).

When several nonlabeled DNA fragments were examined in competition assays, LE9 and LE11 sequences competed to a similar degree against the labeled LE11 probe (Fig. 3C). In a reciprocal experiment using a labeled LE9

probe, the LE9 and LE11 fragments again showed similar competition abilities (data not shown). An "optimal" binding sequence of the Pax6 paired domain (PRD; Fig. 3C) exhibited higher competition ability. These data further support the notion that Pax6 protein, but not Pax6b, can associate directly with LE11 via its paired domain.

Close inspection of the sequence of LE11 enabled us to identify a sequence similar to the consensus recognition sequence of the Pax6 paired domain (Fig. 3A). A series of substitutional mutations in this region (Fig. 3A) were assayed for their competition ability against the wild-type LE11 probe in EMSA. As shown in Fig. 3D, mutants M3 and M4 lost almost all competition ability, whereas M1, M2, and M5 showed various levels of competition ability. Accordingly, when mutant M2 was used as labeled probe, a weak band was detected (Fig. 3B). The above results suggest that the distal half of LE9 (LE11) is the most critical for sequence recognition by Pax6 protein. The corresponding enhancer region of the fugu (pufferfish) *Pax6* gene differs at several positions from those of mouse and human *Pax6* genes when these regions are aligned (Fig. 3A). Oligonucleotides with the fugu sequence (LE11-MF; Fig. 3A) exhibited a high competition ability comparable to those of LE9 and LE11 (Fig. 3D). Taken together, these results indicate that mutations in the LE11 sequence impaired DNA–protein complex formation at various levels, and that some deviations from the mammalian sequence are tolerated. This finding also suggests that the interaction of Pax6 protein with this enhancer site is widely conserved among vertebrates.

Sox2 and Sox3 activate the LE9 enhancer synergistically with Pax6

The results described in the above sections indicate that Pax6 protein contributes to the formation of DNA–protein complexes with LE11 and LE9 in vitro. During the course of these experiments, we noticed that Sox2 and Sox3 can activate the head surface ectoderm-specific enhancer of *Pax6* synergistically with Pax6, and the minimal region for this activation was mapped within LE9. Sox2 and Sox3 proteins are *Sry*-related HMG domain-containing transcription factors and dynamically expressed during lens development (Uchikawa et al., 1999; Baker and Bronner-Fraser, 2001). Since Sox2 and Sox3 are known to modulate enhancer activities through cooperation with partner transcription factors (reviewed in Kamachi et al., 2000), and since the LE9 sequence has multiple copies of HMG domain binding motif-like sequences (Denny et al., 1992), we examined the effects of Sox2 and Sox3 on various fragments of the ectoderm-specific enhancer of *Pax6*. In Fig. 4A, we arbitrarily numbered these three HMG domain binding motif-like sequences according to their order of proximity to the Pax6-binding site, which is indicated by an open box.

When coexpressed with Pax6, Sox2 elevated LE9 enhancer activity synergistically with Pax6 in RL-34 rat liver epithelial cells (Fig. 4B). Similar pattern of activation was observed with other cell lines. Sox2 protein alone resulted in no significant activation of LE9 or other enhancer fragments. Interestingly, Sox2 coexpression caused a detrimental effect on the Pax6-dependent activation of PRDx4. The LE11x4 reporter plasmid also showed decreased activity when Sox2 was coexpressed. Thus, LE11 resembled PRD rather than LE9 in terms of the synergistic effect by Pax6 and Sox2. Since LE11 contains the presumed Pax6 binding sequence, but lacks the HMG domain binding motif-like sequences, these results suggest that the synergistic activation by Sox2 is dependent on the HMG domain binding motif-like sequences within LE9. Sox3 and Pax6 proteins also exhibited a similar synergistic effect (Fig. 4C).

Sequence requirement for the synergistic activation

To determine which part of the sequence is required for the synergistic activation, we introduced a series of mutations (Fig. 4A) within the LE9 or LE6 sequence. As expected from the EMSA (Fig. 3), mutations within the presumed Pax6-binding site (M1, M2, M3, and M4; Fig. 4A) severely diminished the synergistic activation by Pax6 and Sox2 as well as the Pax6-dependent response of the reporter constructs (Fig. 4D). In contrast, mutations (from M5 to M11) located outside of the presumed Pax6-binding site did not affect the ability to show Pax6-dependent activation. These data clearly demonstrate that the Pax6 recognition sequence is essential for the activation of LE9 by Pax6 and for the synergistic activation by Pax6 and Sox2.

Among the mutations outside of the presumed Pax6-binding site, M6, M7, and M11 prevented the Sox2-dependent activation of the LE9 enhancer (Fig. 4D). Thus, among the three HMG domain binding motif-like sequences, the second one was found to be the most important for the synergistic activation of LE9 by Sox2 and Pax6, although other sites might contribute weakly.

The above results identify Sox2 and Sox3 as candidate factors that differentially modify the activity of Pax6 and indicate that their action is dependent on the presumed HMG domain-binding motifs in the LE9 sequence. We therefore performed EMSA using a recombinant Sox2 protein containing the HMG domain and detected a specifically shifted band (Fig. 5A). When nonlabeled competitors with the sequence from the second HMG domain-binding motif (LE15; Fig. 5B) were included, the wild-type sequence, but not mutant sequences, competed well against the LE9 probe (Fig. 5A). LE15 also competed effectively against the DC5 sequence of the chicken δ1-crystallin gene (data not shown), which contains a functional binding site of the Sox2 HMG domain (Kamachi et al. 2001). When Pax6 and Sox2 recombinant proteins were added together with the LE9 probe in EMSA, a supershifted band was detected (Fig. 5C), indicating that these two proteins can bind to LE9 simultaneously under these conditions. Taken together, these results indicate that Sox2 can bind to the proximal half of LE9.

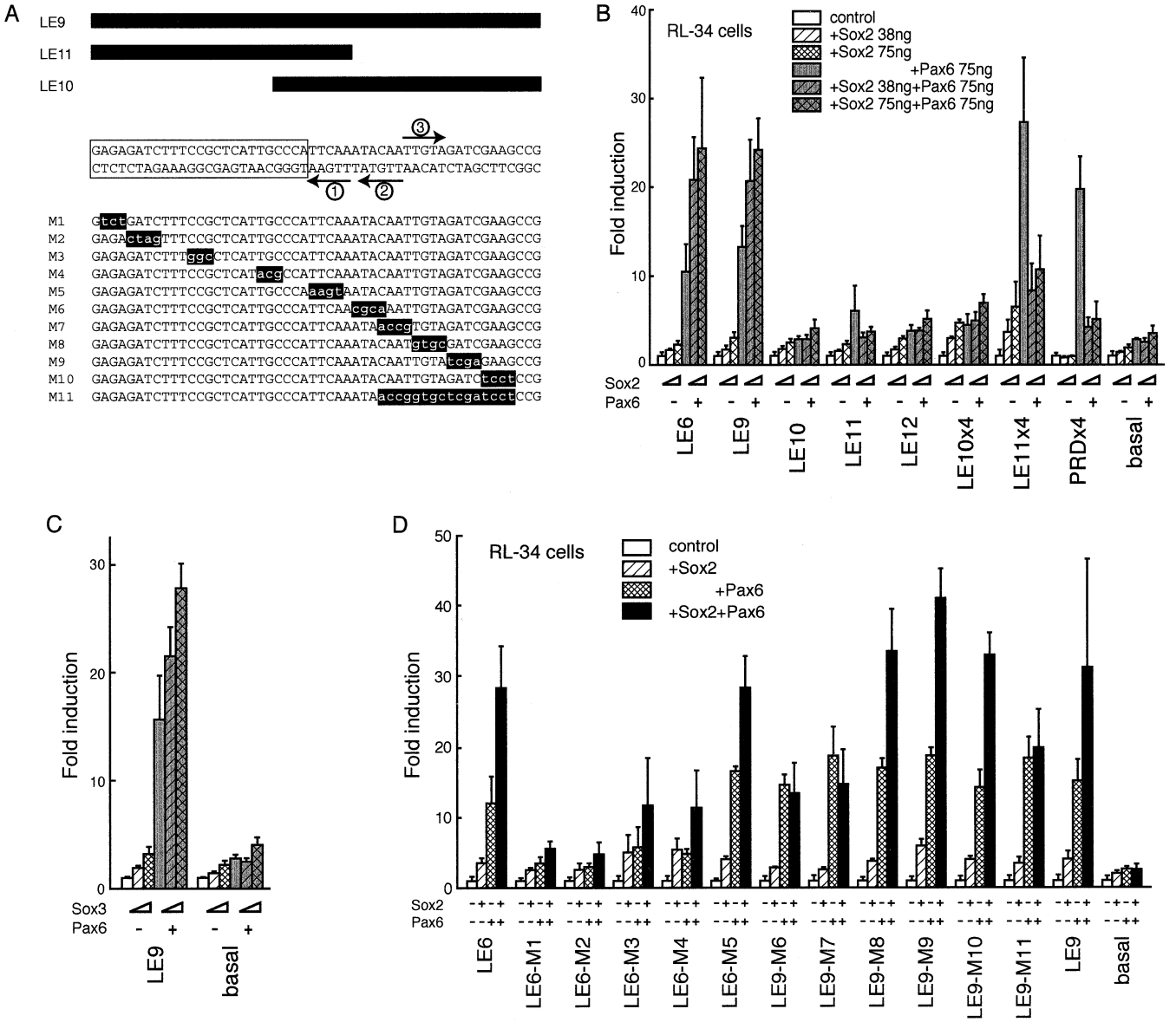

Fig. 4. Pax6 protein acts synergistically with Sox2 and Sox3 proteins to activate the LE9 enhancer of the *Pax6* gene. (A) Candidate sites for the Sox protein recognition within the LE9 enhancer sequence. The sequence of LE9 (double stranded) is presented and the positions of overlapping enhancer fragments (LE9, LE10, and LE11) are schematically indicated by black boxes. The putative Pax6-binding sequence identified in this study is indicated by an open box. Three HMG-domain binding motif-like sequences (TTGTA or TTGAA) are arbitrarily numbered and marked by arrows indicating the orientation. The LE9 regions of reporter plasmids with mutated enhancer sequences are shown in an alignment. The introduced nucleotide substitutions are indicated by white letters on black rectangles. (B) Synergistic activation of LE9 by Pax6 and Sox2 proteins. RL-34 cells were transfected with each indicated reporter plasmid and increasing amounts of the Sox2 expression construct (0, 38, or 75 ng per well of a 12-well plate) in the absence or presence of the Pax6 expression plasmid (0 or 75 ng). The activity of each reporter alone was taken as 1. LE9 reporter was synergistically activated by Pax6 and Sox2, whereas Sox2 had detrimental effects on the activation of LE11 and the "optimal" Pax6 binding sequence (LE11x4 and PRDx4, respectively) by Pax6 protein. (C) Synergistic effect of Sox3 and Pax6 protein. Sox3 expression vector and either control vector or the Pax6 expression vector were cotransfected with LE9 reporter plasmid. Sox3 showed a pattern of synergistic activation similar to that of Sox2. (D) Sequence requirement for the synergistic effects of Pax6 and Sox2. Reporter plasmids with the mutated enhancers listed in (A) were assayed for transactivation by Pax6 and Sox2 via transfection of 75 ng of each expression plasmid. The activity of each reporter without the expression plasmids was regarded as 1.

Complex formation between Pax6 and Sox2 proteins

To examine whether Pax6 protein and Sox2 protein interact physically, we produced Flag-tagged Pax6 and Myc-tagged Sox2 and performed immunoprecipitation assays using anti-Flag and anti-Myc monoclonal antibodies. These two tagged proteins exhibited synergistic activation of LE9 similar to that of untagged Pax6 and Sox2 in reporter assays (data not shown).

Initially, Flag-Pax6 and/or Myc-Sox2 were expressed and cellular proteins were immunoprecipitated by using anti-Myc antibody. The collected immune complex was

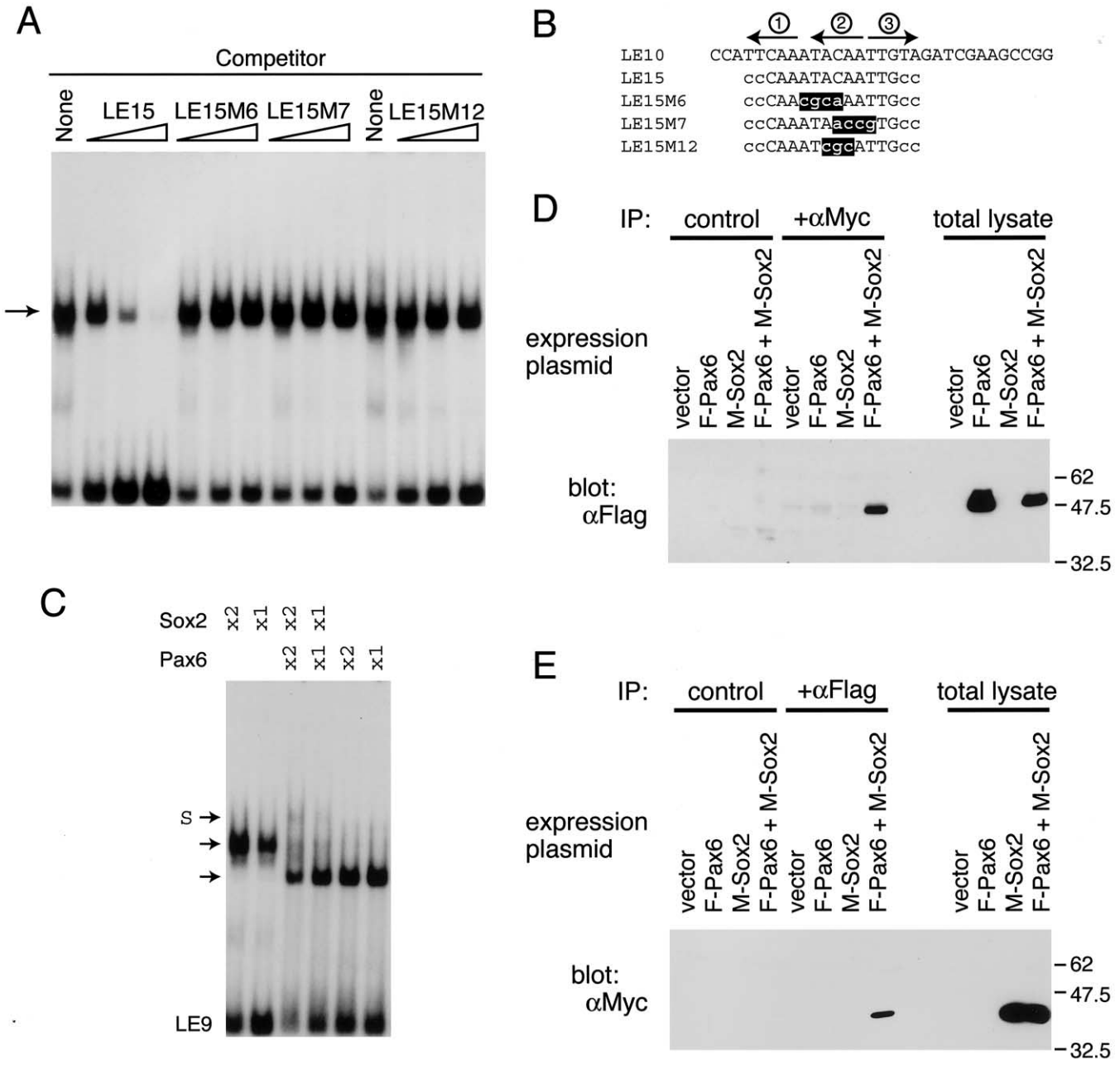

Fig. 5. Sox2 forms a complex with LE9, and interacts with Pax6. (A) Complex formation with ^{32}P-labeled LE9 probe and recombinant Sox2 protein containing the HMG domain (indicated by an arrow). Various amounts of nonlabeled competitors (0.13, 0.66, and 3.3×10^{-12} mol) were included in the EMSA. (B) Sequences of the competitors used in (A). LE15 contains the second candidate sequence for Sox2 binding. Mutations M6 and M7 correspond to those used in the reporter assays in Fig. 4. Three HMG domain-binding motif like sequences are marked by arrows. (C) Complex formation of Pax6 and Sox2 proteins with ^{32}P-labeled LE9. Recombinant Pax6 protein containing the paired domain was included at 0,100 (labeled as x1), or 200 ng/ml (x2). Recombinant Sox2 protein was added at 0, 40 (x1), or 80 ng/ml (x2). Shifted bands are indicated by arrows. When recombinant Pax6 and Sox2 proteins were included together, a supershifted band appeared (indicated by "S"). (D) Flag-tagged Pax6 present in the immunoprecipitate for Myc-tagged Sox2. Lysates from cells transfected with expression vector(s) encoding Flag-tagged Pax6 (F-Pax6), Myc-tagged Sox2 (M-Sox2), or both were incubated with control IgG or an anti-Myc monoclonal antibody. The collected immune complexes were analyzed by Western blotting using an anti-Flag monoclonal antibody. As controls, diluted whole cell lysates were also subjected to Western blotting. The sizes of marker proteins in kilodalton (kDa) and their positions are shown on the right side. (E) Myc–Sox2 present in the immunoprecipitate for Flag-Pax6. In a reciprocal experiment, the cell lysates were immunoprecipitated with an anti-Flag antibody or control IgG, and then the immune complexes were analyzed by Western blotting using an anti-Myc monoclonal antibody.

resolved by SDS–PAGE and subsequently blotted by using anti-Flag antibody. A specific band for Flag–Pax6 was observed only when Flag–Pax6 and Myc–Sox2 were coexpressed (Fig. 5D). In a reciprocal experiment with anti-Flag antibody immunoprecipitation followed by anti-Myc antibody immunoblotting, a specific band for Myc–Sox2 was

detected (Fig. 5E). Thus, Pax6 and Sox2 proteins can form a complex under these conditions.

Discussion

In this study, we identified and mapped a Pax6-responsive element (LE9) within the head surface ectoderm-specific enhancer of the mouse *Pax6* gene. Sox2 and Sox3 proteins activated the LE9 reporter construct synergistically with Pax6. Several lines of evidence suggest that Pax6 protein binds to the distal half of LE9 (LE11) directly via its paired domain: (1) Pax6b protein was inactive in both the reporter assay and the EMSA. (2) LE11 has sequence similarity to the Pax6 paired domain-binding consensus. (3) Oligonucleotides with an "optimal" paired domain-binding sequence of Pax6 competed with this element effectively in the EMSA. This DNA–protein interaction may be evolutionarily conserved in vertebrates, since the corresponding region from the pufferfish *Pax6* gene showed strong competitive activity with the mouse element in the EMSA.

The recognition sequences of the paired domains of the Pax family proteins are unusually long (up to 20 bp in length) and seemingly degenerate. Pax6-binding sites have been mapped in the regulatory regions of several crystallin genes, and they deviate from the consensus sequence deduced from the PCR-based random oligonucleotide selection method (Cvekl et al., 1995; Cvekl and Piatigorsky, 1996; Epstein et al., 1994a). Naturally occurring Pax5-binding sites present in Pax5-target genes deviate at several positions from the consensus binding sequence of Pax5, and they usually bind the paired domain of Pax5 with lower affinities than sequences matching the consensus (Czerny et al., 1993; Czerny and Busslinger, 1995). These observations suggest that the high affinities which consensus sequences possess are not always necessary in vivo (Czerny and Busslinger, 1995), and that it is very difficult to identify a Pax-binding element based only on sequence information. Multiple lines of evidence, such as from reporter assays and EMSAs, are necessary for identifying an actual target site of a Pax protein.

Several reports have indicated that Pax6 protein itself is essential for the maintenance of *Pax6* gene expression in the developing lens in vivo (Grindley et al., 1995; Ashery-Padan et al., 2000). By conditionally disrupting the *Pax6* gene in the presumptive lens ectoderm using the Cre-loxP system, Ashery-Padan et al. (2000) showed that the head surface ectoderm-specific enhancer activity in this area is lost without the presence of Pax6 protein. Together with detailed analyses of chimeric mouse embryos consisting of $Pax6^{-/-}$ and wild-type cells (Quinn et al., 1996; Collinson et al., 2000), those reports indicated that *Pax6* positively autoregulates itself during lens development in a cell-autonomous fashion. Our findings in the present study suggest that direct interaction of Pax6 protein with the head surface ectoderm-specific enhancer contributes to this *Pax6* autoregulation.

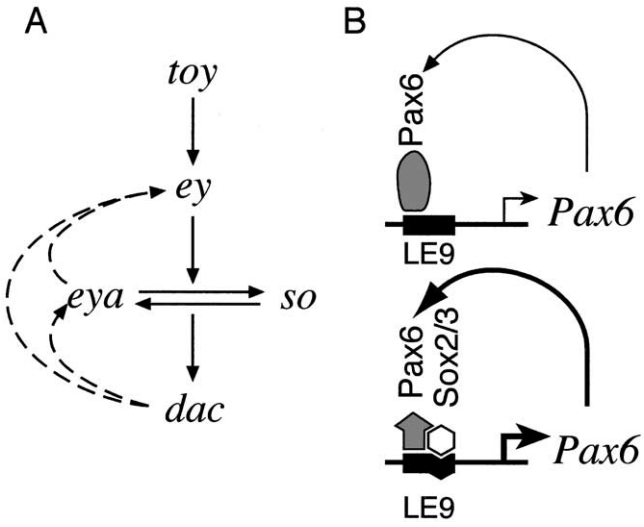

Fig. 6. A model for the *Pax6* expression in the lens. (A) The *ey/so/ea/dac* genetic network identified by *Drosophila* genetic analyses (reviewed in Treisman, 1999). (B) A model for direct autoregulation of the *Pax6* gene in the lens proposed based on this study. In this model, Pax6 protein binds to the LE9 region in the head surface ectoderm-specific enhancer of the *Pax6* gene, and positively regulates its own expression. Pax6 protein also interacts with Sox2 and Sox3 proteins to activate LE9 synergistically. Autoregulation of *Pax6* may be homologous to the activation of *ey* by *toy*, the latter of which is closely related to *ey*.

Our results agree well with the in vivo findings discussed above, but of course do not exclude the possibility of indirect autoregulation of the *Pax6* gene expression. *Drosophila* genetic analyses have identified *eyeless (ey)* and *twin of eyeless (toy)*, both of which are homologues of *Pax6*, as well as the *sines oculis (so)*, *eyes absent (eya)*, and *dachshund (dac)* genetic network in which these genes are interdependently activated (Fig. 6A; reviewed in Treisman, 1999). Since mammalian homologues of the *so*, *eya*, and *dac* genes have been identified and some of these homologues are known to be expressed in the lens area, Pax6 may indirectly autoregulate its expression through a similar genetic network. Indeed, this type of regulation may be conserved in retina development, since the control region of the *Drosophila ey* gene has been found to direct reporter gene expression in the retina of transgenic mice (Xu et al., 1999). To our knowledge, however, there has been no report showing that the homologues of the *so*, *eya*, and *dac* genes transactivate the head surface ectoderm-specific enhancer of the *Pax6* gene. In addition to the direct autoregulatory mechanism proposed here, it will be important to examine whether an indirect mechanism also mediates another level of regulation of Pax6 expression and function. The Pax6b isoform, which plays important roles in eye development (Singh et al., 2002), might be involved in such regulation, although this isoform neither binds to nor stimulates the LE9 enhancer of the *Pax6* gene. It is noteworthy that the direct autoregulation of *Pax6* proposed in this study may be

homologous to the activation of *ey* by *toy*, the latter of which is closely related to *ey* and *Pax6*.

Our results indicated that Sox2 and Sox3 can modify the transcriptional activity of Pax6 protein differentially in a sequence-dependent manner. Sox family proteins are known to modulate enhancer activities through cooperation with partner transcription factors, and the number of reported partner factors is quite large (reviewed in Kamachi et al., 2000). Furthermore, a growing number of transcription factors have been shown to interact with Pax6 or other members of the Pax family. These include Ets and Pax5 for synergistic interaction with the binding sequences in the *mb-1* gene promoter region, and Sox10 and Pax3 for the *MITF* and c-*ret* genes (Bondurand et al., 2000; Potterf et al., 2000; Lang et al., 2000). The synergistic interaction of Pax6 protein with Sox2 or other proteins thus might be involved in the regulation of a variety of genes. Recently, Kamachi et al. (2001) reported that Sox2 and Pax6 proteins form a ternary complex with an enhancer element termed DC5 of the chicken δ1-crystallin gene, resulting in cooperative activation of the enhancer fragment. LE9 in the present study differs from DC5 in several respects: (1) In contrast to DC5, LE9 is activated substantially by Pax6 alone in reporter assays; (2) the orientation of the second HMG binding motif in LE9 relative to the Pax6 binding site is opposite from that of DC5. Pax6 protein seems to bind to LE9 strongly without cooperative binding with Sox2, since in EMSA, Pax6-LE9 interaction is detected in the presence of a high concentration of nonspecific DNA, whereas Pax6–DC5 interaction is inhibited (data not shown). Thus, Sox2 and Sox3 may affect the LE9 enhancer activity via other molecular mechanisms, such as DNA-bending.

We showed here that, in addition to the proposed Pax6-recognition site, the HMG domain binding motif-like sequences in LE9 are essential for the synergistic activation by Pax6 and Sox2/3. On the other hand, Sox2 and Sox3 had detrimental effects on the ability of Pax6 to activate the Pax6-binding sites (LE11 and PRD) lacking the HMG domain binding motif-like sequences in reporter assays. Sox2 and Sox3 proteins might recruit Pax6 protein away from the Pax6-binding sites, since the Sox2 and Pax6 proteins can form a complex, as indicated by our immunoprecipitation analyses. It will be interesting to examine whether Sox2 and Sox3 can negatively modulate the expression levels of *Pax6* target genes.

Based on the findings of the present study, we propose a model for the autoregulation of *Pax6* expression in the lens area, in which Sox2 and Sox3 proteins act as modifiers that positively and negatively modulate the Pax6 functions in the genetic cascade of lens development (Fig. 6B). Broad expression of *Pax6* in the head surface ectoderm is detected in the presumptive lens area earlier than the emergence of the lens placode, but later its expression becomes more dominant in the lens placode and the lens vesicle. The levels of expression of *Sox2* and *Sox3* are highly upregulated in the emerging lens placode and also in other sensory placodes (reviewed in Baker and Bronner-Fraser, 2001). Therefore, Pax6 protein may directly contribute to the autoregulation of the *Pax6* gene in the developing lens, whereas Sox2 and Sox3 proteins may be involved in regulating the expression of *Pax6* and other genes during and after the placode stage of lens development, as illustrated in Fig. 6B.

In our model, *Sox2* and *Sox3* are placed in the direct upstream position of *Pax6* gene expression. This model is consistent with the results of an in vivo study in which *Sox3* was overexpressed in medaka fish embryos (Köster et al., 2000). Those experiments revealed that ectopic *Sox3* expression leads to ectopic expression of endogenous *Pax6* and *Eya1* (a homologue of the *Drosophila eyes absent* gene that is expressed in the otic placode and vesicle) genes in the embryonic ectoderm and causes formation of ectopic lens tissues and otic vesicles, respectively. Those authors observed spatial restriction of gene activation: ectopic *Pax6* expression and subsequent formation of ectopic lenses exclusively in the ventrolateral head ectoderm, which overlaps with the region known to have competence or bias for lens placode formation (Grainger, 1992), and ectopic *Eya1* expression in the posterior and dorsal head- and trunk-ectoderm. Our model can explain how ectopic *Sox3* expression activates ectopic *Pax6* expression in a regionally restricted manner, i.e., due to the requirement for preceding *Pax6* expression.

Pax6 is expressed in several tissues in addition to the lens, such as the retina, the neural tube, and the pancreas. *Sox2* or *Sox3* expression overlaps with *Pax6* expression in the lens and several other tissues, most notably in the retina and the neural tube (Baker and Bronner-Fraser, 2001). Although it is unclear at present why the head surface ectoderm-specific enhancer of the mouse *Pax6* gene is inactive in these tissues other than the lens, one possible explanation is that the enhancer region contains negative regulatory elements that suppress the proposed positive feedback loop in these tissues. It is noteworthy that we observed negative effects of the region outside of LE9 in the reporter assays (Figs. 1 and 2). Kammandel et al. (1999) reported ectopic expression of the *lacZ* reporter in the retina when some parts (outside of LE0 in Fig. 1) of the enhancer region were deleted in the transgenic mouse study. Those authors therefore postulated the presence of negative regulatory elements outside of LE0. Furthermore, Plaza et al. (1999) reported that their reporter constructs carrying parts of the "B fragment" of the quail *Pax6* gene enhancer region, which corresponds to the head surface ectoderm-specific enhancer of mouse, are active in the reporter assays using cultured embryonic neuroretina cells. Interestingly, their shortest fragment (corresponding approximately to LE2 in this report) showed a higher activity than other longer enhancer constructs. These findings support the notion that negative regulatory mechanisms also play important roles in the tissue specificity of the head surface ectoderm-specific enhancer of the *Pax6* gene.

The level of *Pax6* expression in the lens area is crucial

for lens development, and the mechanisms regulating this expression are considered to be complex and multistep. Our results will provide important clues to understanding the molecular mechanisms of lens development.

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